Chapter 6

Discussion

Recently, precision-cut liver slices came into notice as a potential model for the study of stellate cell activation and liver fibrosis because, unlike current *in vitro* models, they could enable to study these processes *in vitro* in a multicellular system in which cell-cell and cell-extracellular matrix interactions are maintained. Precision-cut liver slices are extensively used for the study of drug metabolism and toxicity [1, 2], however, studies describing liver slices as a tool to study hepatic stellate cell activation and fibrogenesis are still limited. Therefore the aim of the research described in this thesis was to evaluate the use of precision-cut liver slices for the study of liver fibrosis and as a test-system for anti-fibrotic compounds.

1 Preparation and culturing of liver slices

To obtain precision-cut slices, tissue cores with a diameter of 5-8 mm are made that are subsequently placed in a tissue slicer to be cut into slices with a reproducible thickness. Two tissue slicers are developed for this purpose, the Krumdieck tissue slicer and the Brendel-Vitron tissue slicer, which in general perform equally well [1]. Ideally, liver slices should have a thickness of less than 250 µm to allow oxygen and nutrients to diffuse to the inner cell layers, but more than 175 µm to keep the ratio of damaged cells at the outer layers to the living cell mass as favorable as possible [2]. However, recent research showed that for studies on hepatic metabolism even slices with a thickness of 100 µm could be used successfully [3].
Various systems for the culturing of precision-cut liver slices are in use, which can be divided in continuously submerged culture systems and dynamic organ culture systems [2]. In continuously submerged culture systems the slices are floating within the culture medium in 6-, 12- or 24-wells-plates or in flasks while the system is gently shaken, or the slices are placed on a stainless-steel grid while the culture medium is magnetically stirred. In the dynamic organ culture system slices are alternately exposed to the gas phase and the culture medium by placing the slices on inserts in a glass vial or 6-wells culture plate, which is rolled or rocked, respectively, during incubation. Irrespective of the culture system, the liver slices are incubated at 37°C in a humidified incubator in the presence of oxygen concentrations varying between 95% oxygen/5% CO_2 and 20% oxygen (air)/5% CO_2. It was suggested that for short-term studies, incubation in 20% O_2/5% CO_2 is sufficient to retain slice viability [1], whereas oxygen concentrations of at least 40% are essential for prolonged incubation of liver slices [2, 4]. In addition, for long-term incubation, nutrient-rich culture medium is required.
There is no clear consensus regarding the maximum time that liver slices can be cultured while maintaining slice viability, mainly due to differences in incubation methods and viability parameters used. For incubations up to 24 hours, provided that oxygen concentrations are high enough, slice viability is retained equally well in the incubation systems described above, with the exception of the stirred 24-well system in which the liver slices showed a decreased viability [5]. Several studies are described using longer incubation times than 24 hours [6-9], but no thorough comparison has been made on the influence of culture conditions on slice viability after longer incubation intervals. It was suggested that for prolonged incubation a dynamic organ

culture system as well as enriched culture medium are needed to retain slice viability [1, 6, 10]. With optimal culture conditions, currently liver slices remain viable in culture for 3-4 days.
In the studies described in this thesis, two incubation systems were used. The 6-wells shaken culture plate, incubated at 37°C with 95% O_2/5% CO_2 with standard cell culturing medium supplemented with glucose and gentamycin was used for standard incubation. In this system the liver slices remained viable during incubation for 24-48 hours with decreasing viability when incubation was continued. In case of incubations with carbon tetrachloride, which is a very volatile toxic compound, the use of an air-closed system was necessary. Shaken Erlenmeyer flasks were used for this purpose. This incubation system showed similar retention of slice viability as the 6-wells plates during 24 hours of incubation [5]. Although these systems were suitable for the present studies, after prolonged incubation (48-72 hours) viability of the liver slices decreased. Whether this can be ascribed to the relatively poor culture medium or the incubation systems used needs further investigation.

2 Liver slices as *in vitro* model for the (diseased) liver

2.1 CELL FUNCTIONALITY AND INTERCELLULAR INTERACTIONS
Several studies on the viability and functionality of the different cell types present in liver slices have shown intact cellular functions of hepatocytes [1, 11], Kupffer cells [11-13], endothelial cells [14], and hepatic stellate cells (chapter 2 and 3 and [15]) during incubation. The presence and functionality of all liver cell types provide the possibility to study the liver physiology and pathophysiology *in vitro* in a multicellular context. Importantly, unlike co-culture models of isolated cells, the cells are maintained in their original extracellular matrix, with the same relative cell number and orientation towards other cells. In addition, the acinar structure of the liver is maintained. However, when used for prolonged incubation it should be noted that during culturing of liver slices after 48 hours of incubation some degree of cell proliferation may occur [6]. Different regenerative capacities and proliferation rates of several liver cell types could result in an altered cellular composition of the liver slices. Besides, the demonstration of certain intact cellular functions does not imply that all cellular functions are preserved during culture of the liver slices. For instance the drug metabolism activity of hepatocytes in liver slices shows a time-dependent decrease during incubation, in particular for cytochrome P450 mediated processes [16, 17], as is also observed in hepatocyte cell cultures [17]. In addition, although the different cell types remain functionally active in the liver slices, their activities could change during incubation, which is for example reflected by the increased expression of fibrogenic markers during incubation of rat and human liver slices (chapter 4 and 5). Nevertheless, the presence and functionality of all liver cell types in their original, three-dimensional multicellular milieu is one of the main advantages of the use of liver slices over the use of cell culture models. This feature of liver slices allows the testing of multicellular processes and interactions in the liver in a milieu closely resembling the *in vivo* situation. Indeed, a number of studies have demonstrated intact interactions between

Kupffer cells and hepatocytes in the liver slice [11-13]. In addition, the carbon tetrachloride-induced early activation of hepatic stellate cells in liver slices (chapter 2 and 3) is also most likely caused by a multicellular process, which besides stellate cells involves both hepatocytes and Kupffer cells. Liver slices thus provide an *in vitro* system that closely resembles the *in vivo* situation in the liver.

2.2 THE EXTRACELLULAR MATRIX

The extracellular matrix in the liver consists of several types of collagens, glycoproteins, and proteoglycans, which surround the different liver cell types and thereby both assure the coherence of the liver and regulates cellular functions. The composition of the extracellular matrix can influence the differentiation, proliferation, and activation state of liver cells [18-20]. This effect of extracellular matrix on cell function is mainly mediated via integrin receptors, which are located on the cell membrane and bind to extracellular matrix molecules [21]. In addition, the extracellular matrix can bind and release several growth factors, cytokines, and hormones, which in turn influence cellular behavior [22, 23].

Given the important role of the extracellular matrix in regulating cellular function, the presence of the physiologic extracellular matrix in liver slices provides a major advantage compared to cell culture models in mimicking the *in vivo* situation of the liver. However, during incubation of liver slices pro-collagen 1a1 mRNA expression and collagen protein content significantly increased (chapter 4 and 5). An increased collagen production in liver slices during incubation and increased expression of genes associated with extracellular matrix deposition was reported previously [6], and suggests that the extracellular matrix in the liver slice may be subject to alteration. Because the protein composition of the extracellular matrix can influence cell function, this should be taken into account in liver slice studies.

2.3 THE IMMUNE SYSTEM AND BONE MARROW DERIVED CELLS

The liver has an important function in the defense against pathogens and is involved in both the innate and the adaptive immune response. Its involvement in the innate immune response consists of non-specific phagocytosis of antigens and pathogenic microorganisms, and intrahepatic cell killing. For the first process Kupffer cells are responsible, in cooperation with sinusoidal endothelial cells and resident dendritic cells [24, 25]. The latter process is mediated by resident and blood-derived natural killer cells and by natural killer T cells, which have characteristics of both T lymphocytes and macrophages and play a major role in the defense against tumor cells [25, 26]. The involvement of the liver in the adaptive immune response is illustrated by the presence of a liver specific population of T lymphocytes, which is distinct from other T lymphocyte populations [27]. In addition the liver plays an important role in the removal of lymphocytes that were activated at distant parts in the body and is involved in the induction of immune tolerance [24, 25].

Vice versa, the immune system and bone marrow derived cells play a role in the response of the liver to injury. Firstly, injury to the liver results in the attraction of different types of immune cells into the liver and in the activation of resident immune cells. The immune cells infiltrating the liver can include B and T lymphocytes, natural

killer cells, and polymorphonuclear cells, among which the precursors of macrophages. The specific composition of this cellular infiltrate may vary with the type and stage of liver injury [28]. The recruitment and activation of inflammatory cells and their subsequent secretion of inflammatory cytokines influences the response of the liver cells. Secondly, when following liver injury the regenerative capacity of mature cells is impaired, progenitor cells are responsible for liver regeneration. The progenitor cells involved in this process consist of a small population of resident progenitor cells, which expands following liver injury, and possibly also bone marrow derived progenitor cells are involved [29, 30]. Thirdly, recent studies suggest that a significant proportion of hepatic stellate cells in mouse liver is of bone marrow origin [31, 32] and that during the development of liver fibrosis in patients (myo)fibroblasts derived from bone marrow derived progenitor cells contribute to the fibrotic process [33].
With respect to liver slices, several studies showed the functionality of Kupffer cells during culture of rat and human liver slices [11-13]. Similarly also other resident immune cells, such as dendritic cells and lymphocytes, as well as progenitor cells will likely remain present and functional in the liver slice, although this has not been studied. To incorporate also the effects of blood derived immune cells and bone marrow derived cells on processes in the liver slice model, co-cultures of liver slices with these cells can be used.

2.4 THE CENTRAL NERVOUS SYSTEM AND NEUROACTIVE COMPOUNDS

Innervation of the liver is mainly located in the portal area's, although in certain species, among which human, also substantial interlobular innervation is observed [34, 35]. In addition, area's distant from the respective nerves could be under neuronal regulation via diffusion of neurotransmitters, gap junctional communication, or other intercellular interactions. The central nervous system is thought to play a role in the regulation of several processes in the liver, of which the most important are discussed here briefly.
Firstly, the maintenance of metabolic homeostasis, which is the main function of the liver, is under tight neuro-hormonal regulation. However, although several studies have shown the influence of direct stimulation of autonomic nerves on different aspects of metabolism [36], the importance of direct nerve stimulation in regulating hepatic metabolism might be limited and is likely much less important than the regulation by circulating metabolite levels and hormones. This is especially clear when considering that metabolic homeostasis after denervation of the liver due to transplantation is well maintained. On the other hand, it was suggested that in the development of metabolic diseases, hepatic innervation might play an important role [36].
Secondly, the function of local immune cells can be modulated by the central nervous system. It was suggested that phagocytosis and antigen presentation by dendritic cells, T lymphocyte function, and probably also Kupffer cell function, could be modulated by the nervous system [37]. This conclusion was based on the close proximity of nerve fibers to hepatic immune cells and on the presence of receptors for neuroactive compounds on these cells. In addition, several studies in *in vivo* models for hepatitis indicated neuronal modulation of the immune response [37, 38].

Thirdly, the contractility of hepatic stellate cells and regulation of the microcirculation of the liver is mediated by several neurotransmitters in cooperation with vasoactive compounds [39].
Finally, the autonomic nervous system appears to play an important role during injury and repair processes in the liver [40, 41]. In this respect, studies have been mainly focused on the activation of the sympathetic nervous system, which aggravates several types of liver injury [42-44], influences regeneration of the liver after injury [42, 45], and promotes the development of fibrosis [41, 45]. In addition, inhibition of the sympathetic nervous system inhibited fibrogenesis in an experimental animal model for liver fibrosis [46]. The parasympathetic nervous system was shown to influence the regeneration process [29], but was not studied with respect to liver injury or during fibrosis development.
Taken together, these studies indicate that, especially when studying inflammation and injury and repair processes in the liver, it may be important to incorporate effects of the central nervous system. Although in liver slices innervation is lost, responsiveness of the different liver cell types in the slice to neurotransmitters and other neuroactive compounds is likely maintained, providing the opportunity to include this aspect of liver functioning in a multicellular system *in vitro*.

3 Liver slices as *in vitro* model for liver fibrosis

The development of liver fibrosis *in vivo* is a multicellular process that occurs in response to chronic liver injury, as is described in more detail in chapter 1. Briefly, injured liver cells produce several mediators that initiate the activation of hepatic stellate cells and/or other fibrogenic cells and the production of excess extracellular matrix. In addition, liver injury leads to the recruitment of immune cells into the liver and to the activation of local Kupffer cells, which can further promote the fibrotic process [23, 47]. The increased deposition of extracellular matrix is mainly caused by the increased production of matrix proteins by activated stellate cells. In addition, matrix breakdown is decreased due to alterations in the expression levels of metalloproteinases and their inhibitors. These processes result in accumulation of extracellular matrix and in profound changes in its protein composition, which in turn can further promote the activation and proliferation of stellate cells and thus contribute to the progression of fibrosis [48, 49]. The extent of fibrogenesis and inflammation depends on the type and duration of the liver injury. When injury is acute, the fibrotic response is taken over by regeneration, with replacement of apoptotic or necrotic cells, removal of scar tissue, and resolution of inflammation. However, when injury is sustained, the regeneration process is insufficient and liver tissue is gradually replaced by extracellular matrix.
Given the above-described mechanism of fibrosis development in the liver, liver slices may provide a promising tool for the study of liver fibrosis *in vitro*, especially when compared to current available *in vitro* models, which encompass cell culture models of primary cells or cell lines. As liver slices can at least partially account for the influence of the immune system and central nervous system in the development of liver fibrosis,

they represent a potent *ex vivo* model filling the gap between *in vivo* and cell culture models. Liver slices provide a multicellular system where cell-cell and cell-extracellular matrix interactions, which play an important role in the development of liver fibrosis, are maintained.

3.1 MEASURING FIBROSIS IN LIVER SLICES

Activation of hepatic stellate cells is generally considered to be the key event in the development of liver fibrosis. Therefore, to study the applicability of liver slices for the study of fibrosis, the primary focus was on the hepatic stellate cell. The main functions of hepatic stellate cells in a healthy liver are the uptake, storage, and release of retinoids, and the synthesis and degradation of extracellular matrix [50]. During liver fibrosis, hepatic stellate cells become activated and transform into myofibroblasts-like cells, which are the main producers of the excess extracellular matrix leading to liver dysfunction [47]. Thus, increased hepatic stellate cell activation in liver slices was considered as an indication for the early manifestation of fibrosis. To detect stellate cell activation in the liver slices, stellate cell specific markers were studied using real-time PCR to assess mRNA expression, and Western Blot analysis to determine protein expression. In addition, attempts were made to determine protein expression levels using immunohistochemical methods. This would provide additional information about the localization of the cells expressing the marker proteins in the liver slice, which is often the only way to differentiate activated hepatic stellate cells from other (myo)fibroblasts in the liver. In this respect, the use of laser capture microscopy to specifically isolate hepatic stellate cells or other liver cell types from the liver slice and study gene and protein expression in the specific cell types, was also explored.

Several cellular markers can discern hepatic stellate cells from hepatocytes, Kupffer cells, and endothelial cells in normal liver. Among these markers are the cytoskeletal proteins desmin and glial fibrillary acidic protein, heat shock protein 47 (HSP47), the retinol-handling protein retinaldehyde dehydrogenase 1, and the neuronal protein synaptophysin [50]. When hepatic stellate cells become activated, phenotypic and genotypic alterations take place resulting in a different expression pattern of cellular markers. Most of the markers that are present in quiescent hepatic stellate cells are also expressed in activated cells. Some of these markers, such as desmin in rat liver [51] and HSP47 in both rat and human liver [52-54], show increased expression in activated hepatic stellate cells as compared to quiescent cells. Other markers, like synaptophysin [55] have a more constant expression pattern in both rat and human stellate cells. In addition, some cellular markers are expressed in activated stellate cells that are not, or rarely present in quiescent hepatic stellate cells, like αB-crystallin [56, 57] and α smooth muscle actin (αSMA) in rat liver [50, 51]. In human liver, αSMA and αB-crystallin are also expressed in some stellate cells in normal liver, however, expression is more pronounced in activated human stellate cells in culture and in fibrotic human liver [50, 56]. It is not clear whether the stellate cells in normal human liver that express αB-crystallin and αSMA should be considered to be quiescent or activated. Table 1 gives an overview of the cellular markers that were used in the research

described in this thesis, and indicates the relative expression levels in quiescent and activated stellate cells in human and rat liver.
In theory, increased expression of HSP47, αB-crystallin, desmin, and αSMA in liver slices can be used as a marker for stellate cell activation in the slices. However, the markers that are available to detect activated hepatic stellate cells are general (myo)fibroblast markers and consequently are also present in other types of (myo)fibroblasts that are present in the liver. To our knowledge, there is no marker that can specifically indicate the presence of activated hepatic stellate cells without measuring other (myo)fibroblasts or quiescent hepatic stellate cells (table 1). Thus, when studying stellate cell activation in liver slices, it should be taken into account that increased expression of stellate cell activation markers in the liver slice could also reflect the presence of an increased number of (myo)fibroblasts or stellate cells. On the other hand, although the activated hepatic stellate cells play the major role in the development of liver fibrosis, the population of fibrogenic cells involved in this process is likely more heterogeneous and can also include activated portal fibroblasts and possibly second-layer cells located around centrolobular veins and smooth muscle cells [58]. When liver slices are used to study the development of fibrosis and to test anti-fibrotic drugs, they provide the advantage that the response of all fibrogenic cells in the liver can be studied together, in a milieu that closely resembles the *in vivo* situation.

Table 1. *Relative expression of the cellular markers, which were used in the research described in this thesis, in quiescent hepatic stellate cells (q HSC), activated hepatic stellate cells (a HSC), and (myo)fibroblast populations other than activated hepatic stellate cells (MFB) in rat and human liver. nr – not reported.*

		Rat liver			*Human liver*			
		q HSC	*a HSC*	*MFB*	*q HSC*	*a HSC*	*MFB*	*References*
HSP47	Heat shock protein	+	++	++	+	++	++	[52, 53]
αB-crystallin	Heat shock protein	-	++	++	±	++	++	[56, 57, 59]
Desmin	Cytoskeletal protein	+	++	++	-	-	-	[50, 59]
αSMA	Cytoskeletal protein	-	++	++	±	++	++	[50]
Synaptophysin	Membrane protein	+	+	-	+	+	-	[55, 59]
Fibulin-2	ECM protein	-	-	++	nr	nr	nr	[60, 61]

3.2 CARBON TETRACHLORIDE-INDUCED STELLATE CELL ACTIVATION IN LIVER SLICES

In the first part of this thesis two studies are described that aimed to induce hepatic stellate cell activation in human and rat liver slices. For this purpose, the liver slices were incubated with the fibrogenic compound carbon tetrachloride. Rat liver slices were more susceptible to carbon tetrachloride-induced toxicity as compared to human liver slices and were therefore incubated with lower doses (chapter 2 and 3). This difference may be explained by a difference in cytochrome P450 activity, which is higher in rat liver as compared to human liver [62]. Toxicity and fibrogenic activity of carbon tetrachloride requires conversion into a free radical by cytochrome P450 in hepatocytes. The fibrogenic activity of carbon tetrachloride, therefore, has been linked to oxidative damage to hepatocytes, yielding lipid peroxides and other mediators that activate hepatic stellate cells both directly and indirectly via Kupffer cells [63, 64]. Thus, any stellate cell activation occurring in liver slices in response to incubation with carbon tetrachloride likely results from a multicellular process. This illustrates intact intercellular interactions in the liver slices, as has been shown previously [11, 12]. However, although there are no studies that show that carbon tetrachloride exerts direct effects on stellate cells, this has not been ruled out yet.

In general, a stellate cell is considered as completely activated and transformed from a quiescent cell into an activated, myofibroblast-like stellate cell once it expresses αSMA. However, prior to this already several genotypic and phenotypic changes take place that are accompanied by increased expression of early markers for stellate cell activation, such as HSP47 [52-54] and αB-crystallin [56, 57]. When liver slices were incubated with carbon tetrachloride, expression of these two early markers dose-dependently increased in both rat (chapter 2) and human (chapter 3) liver slices, suggesting that early stellate cell activation could be induced. The changes observed in early marker expression in rat liver slices in response to carbon tetrachloride were consistent with those reported during hepatic stellate cell activation and fibrogenesis *in vivo* [54, 65, 66]. However, as discussed above, these markers are expressed in both activated hepatic stellate cells and in other (myo)fibroblast populations in the liver and do not discern activated hepatic stellate cells from other (myo)fibroblasts. Thus, increased expression of HSP47 and αB-crystallin solely is no proof for the occurrence of stellate cell activation, especially considering that αB-crystallin might also be expressed in a minority of hepatocytes in response to cellular stress [56]. Alternatively, the increased expression of these markers may be caused by an increase in the number of quiescent or activated stellate cells or other (myo)fibroblasts present in the liver slices.

To further explore the above-mentioned possibilities, mRNA expression of a number of other markers for stellate cells and (myo)fibroblasts was studied in human liver slices after incubation with carbon tetrachloride (chapter 3 and 4). Firstly, if the increased expression of αB-crystallin and HSP47 is caused by an increased number of (myo)fibroblasts in the liver slices, this would be accompanied by an increased expression of late markers for stellate cell activation, which as mentioned are also expressed in (myo)fibroblasts. However, no significant increase in mRNA expression of αSMA was observed in human liver slices after incubation with carbon tetrachloride. Secondly, to exclude the possibility that the increased expression of αB-crystallin and

HSP47 is due to an increase in the number of stellate cells present, mRNA expression of synaptophysin, which remains relatively constant during activation of stellate cells [55], was studied. Again, no significant increase was observed in response to incubation with carbon tetrachloride, indicating that the number of stellate cells in the liver slices was not significantly changed. Taken together, it was concluded that early hepatic stellate cell activation is induced in liver slices by incubation with carbon tetrachloride. This method could provide a good system to study the mechanisms of (multicellular) processes underlying toxicity-induced early stellate cell activation in rat and human liver tissue. Although in its present form this model cannot account for more progressed stages of fibrogenesis, it is well known that activation of hepatic stellate cells is the key event in the natural process of wound healing and scar tissue formation as well as in the development of liver fibrosis. Thus, the carbon tetrachloride-induced early stellate cell activation in rat and human liver slices likely reflects the onset of fibrogenesis. Whether this model can discern between the wound healing response and pathological fibrogenesis, and whether there is a difference between these processes remains to be elucidated.

After establishing treatment with carbon tetrachloride as a method to induce early hepatic stellate cell activation in liver slices, subsequently we aimed to determine whether this model could be used to test the effects of anti-fibrotic drugs. In rat liver slices, incubation with carbon tetrachloride resulted in a maximum induction of approximately two-fold for HSP47 mRNA expression and of approximately 160-fold for αB-crystallin mRNA expression after 16 hours of incubation (chapter 2). The induction of αB-crystallin provides a large window in which the effects of anti-fibrotic drugs can be measured. However, the use of αB-crystallin as a marker tot test anti-fibrotic effects of drugs is hampered by the fact that an unknown part of αB-crystallin expression might reflect induction in a minority of hepatocytes in response to cellular stress [56]. Indeed, immunohistochemical analysis of rat liver slices incubated with carbon tetrachloride revealed αB-crystallin protein expression in a subset of hepatocytes located around the central veins (not shown). Thus, using this marker, protective effects on hepatocytes and effects on hepatic stellate cell activation cannot easily be discerned. To test the effects of anti-fibrotic compounds on the activation of hepatic stellate cells the specific stellate cell marker HSP47 would be more discriminative. However, the two-fold increase in HSP47 expression caused by carbon tetrachloride in this rat model was thought to provide too small a window to measure the effects of anti-fibrotic compounds accurately. Therefore, several attempts were made to further optimize the model in order to obtain a stellate cell activation marker expression profile providing a larger window in which anti-fibrotic effects could be tested. However, neither prolonged incubation (24-48 hours) with several, single or repeated, doses of carbon tetrachloride, nor the addition of the cytochrome P450 inducer phenobarbital to the incubation system resulted in further induction of HSP47 and αB-crystallin expression or in pronounced induction of late markers for stellate cell activation, such as αSMA and pro-collagen 1a1 (data not shown). From these observations it was concluded that, although this model provides a system to study the mechanisms underlying early stellate cell activation, in its present form, its application as a test system for anti-

fibrotic drugs needs further investigation on specific markers for early stellate cell activation in rat liver.
In contrast to the situation in rat liver slices, carbon tetrachloride induced a pronounced expression of both HSP47 and αB-crystallin mRNA in human liver slices (chapter 3), providing a large window in which effects of anti-fibrotic drugs could be tested. In addition, immunohistochemical analysis of human liver slices incubated with carbon tetrachloride did not show any αB-crystallin protein expression in hepatocytes (not shown). Therefore, this model was further evaluated as a tool to test the effects of anti-fibrotic drugs (chapter 4). For this purpose, human liver slices were incubated simultaneously with carbon tetrachloride and the anti-fibrotic compound pentoxifylline. The anti-fibrotic effects of pentoxifylline are generally attributed to inhibition of platelet-derived growth factor (PDGF) signaling. PDGF is the most potent inducer of hepatic stellate cell proliferation in culture and plays a role in the perpetuation of stellate cell activation [67]. Pentoxifylline can interfere with PDGF signaling via the elevation of intracellular cyclic adenosine mono-phosphate (cAMP) levels due to inhibition of phosphodiesterases [68]. cAMP is an important second messenger molecule and is part of the negative feedback loop of PDGF signaling [23]. Inhibitory effects of pentoxifylline on the downstream pathways of PDGF signaling are indeed reported [69, 70] and several studies showed that pentoxifylline can inhibit PDGF-induced proliferation and collagen synthesis in cultured hepatic stellate cells and (myo)fibroblasts [71-73]. However, it was also shown that pentoxifylline inhibits stellate cell activation independent of its phosphodiesterases inhibitory activity [74]. Therefore, the exact mechanism of action of pentoxifylline remains to be elucidated, and may be not exclusively mediated via interfering with PDGF signaling.
Carbon tetrachloride-induced early stellate cell activation in human liver slices was not inhibited by pentoxifylline, whereas pentoxifylline did inhibit mRNA expression of late markers for stellate cell activation and fibrogenesis in the liver slices (chapter 4). These results suggest that pentoxifylline is unable to inhibit early stellate cell activation. Previously, it was reported that only activated stellate cells express the PDGF receptor and therefore responsiveness of stellate cells to PDGF may require that the cells are fully activated [75]. If the inhibitory effect of pentoxifylline is solely mediated via its effect on PDGF signaling, this could explain why pentoxifylline is unable to inhibit the early stages of hepatic stellate cell activation observed in human liver slices after incubation with carbon tetrachloride. Indeed, after 16 hours of incubation with carbon tetrachloride, PDGF receptor mRNA expression was not increased in human liver slices (chapter 4). Thus, although this model can be used to study effects of anti-fibrotic drugs on early hepatic stellate cell activation in human liver, future studies with other types of inhibitors, which act on different pathways involved in stellate cell activation, are needed to confirm this.

3.3 SPONTANEOUS FIBROGENESIS IN LIVER SLICES

One of the drawbacks of the use of cell cultures as *in vitro* model for liver fibrosis is the spontaneous activation of hepatic stellate cells when cultured on uncoated plastic. This phenomenon makes it difficult to study the activation process of hepatic stellate cells in a systematic manner. To overcome this limitation, several culture matrixes have been developed, which can prevent or even reverse activation of hepatic stellate cells in culture [76-78]. However, matrix proteins by themselves might also influence both parenchymal and non-parenchymal cell behavior [18-20] and the *in vivo* cell-extracellular matrix interactions cannot adequately be mimicked in culture. In contrast, precision-cut liver slices provide an extracellular milieu for the cells that resembles the *in vivo* situation as close as possible with respect to cellular and matrix constituents. Therefore, we hypothesized that stellate cells would remain quiescent during incubation of liver slices.

Indeed, as described in chapter 2, when rat liver slices were cultured for 16 hours, expression of the early markers for stellate cell activation HSP47 and αB-crystallin did not increase, from which it was concluded that the hepatic stellate cells maintained their quiescent phenotype. However, when the incubation time was prolonged, mRNA expression of HSP47 and αB-crystallin (not shown) as well as of αSMA and pro-collagen 1a1 mRNA in rat liver slices started to increase (chapter 5). In human liver slices, an increased mRNA expression of HSP47 and αB-crystallin was observed already in the first hour of incubation and after prolonged incubation expression of αSMA and pro-collagen 1a1 mRNA as well as collagen protein content of the liver slices increased (chapter 4). These changes in expression of fibrogenic markers were described previously in rat liver slices [6] and could indicate activation of stellate cells or fibroblasts, or the presence of an increased number of (activated) stellate cells or (myo)fibroblasts in the liver slices.

To further evaluate the involvement of stellate cells and other (myo)fibroblasts in the fibrotic process that occurred after prolonged incubation of the liver slices, the mRNA expression of fibulin-2 and synaptophysin was determined (chapter 4 and 5). Fibulin-2, which is expressed in (myo)fibroblasts in rat liver but rarely in activated stellate cells [60], showed a similar expression pattern as αSMA during incubation of rat liver slices (chapter 5), indicating that (myo)fibroblast populations other than activated stellate cells are at least partly responsible for the increase in αSMA mRNA expression after prolonged incubation. Synaptophysin mRNA expression, which is specific for stellate cells in rat and human liver [55], also increased after prolonged incubation of rat liver slices (chapter 5), suggesting that the number of stellate cells in the slices starts to increase. In contrast to rat liver slices, fibulin-2 and synaptophysin mRNA expression did not increase during incubation of human liver slices (chapter 4), suggesting that the number of (myo)fibroblasts or (activated) stellate cells in human liver slices did not increase during incubation. However, the inhibitory effect of pentoxifylline on fibulin-2 expression after 24 hours of incubation could indicate some involvement of (myo)fibroblasts in the fibrotic process in human liver slices. It was concluded that a spontaneous fibrotic process takes place in rat and human liver slices after prolonged incubation, which may involve both activated stellate cells and other (myo)fibroblast

populations. This is similar to fibrosis development *in vivo*, which is likely also mediated by different populations of (myo)fibroblasts [58].
The increased expression of αSMA and pro-collagen 1a1 mRNA in human and rat liver slices after prolonged incubation, was preceded by an initial decreased expression of these markers during the first hours of incubation (chapter 4 and 5). This was also described previously in rat liver slices [6] but is yet unexplained. In both human (chapter 4) and rat (chapter 5) liver slices the decreased αSMA and pro-collagen 1a1 mRNA expression was paralleled by a decreased mRNA expression of fibulin-2, suggesting that (myo)fibroblast populations other than activated stellate cells are at least partly involved in this process. In rat liver slices a similar expression pattern was observed for synaptophysin mRNA expression (chapter 5), which suggests that also hepatic stellate cells are involved. In human liver slices no decreased expression of synaptophysin was observed during the first hours of incubation (chapter 4), however, since normal human liver also contains stellate cells that express αSMA [50], this result does not exclude a role for stellate cells.
The occurrence of spontaneous stellate cell activation and fibrogenesis might be regarded as a drawback of liver slices when used for the study of liver fibrosis. However, liver slices still provide a multicellular system with all liver cell types retained in their original extracellular matrix, which is an advantage compared to current *in vitro* models. Stellate cell activation and fibrogenesis occurring in liver slices generated from normal liver tissue, suggests that the induction of these processes is intrinsically related to the preparation or culturing method used. Factors in the method used that might trigger the fibrotic process are ischemia-reperfusion injury, the presence of high oxygen tension during incubation, cell death during incubation, or accumulation of (waste) products in the slices. Pilot experiments suggested that the damage to the cells on the surface caused by the cutting of the slices does not play a major role in triggering the spontaneous fibrotic process in liver slices and that accumulation of bile salts, which is potentially fibrogenic, does not occur during incubation of the liver slices. Additional evaluation of the liver slices is necessary to further elucidate this matter.
Finally, we studied the effects of the anti-fibrotic compound pentoxifylline on the spontaneous fibrotic process in liver slices. Pentoxifylline was previously shown to have inhibitory effects on proliferation and collagen synthesis in cultured hepatic stellate cells and (myo)fibroblasts [70-73, 79], and to reduce hepatic stellate cell activation and proliferation in experimental animal models for fibrosis [74, 80]. In line with these studies, pentoxifylline significantly inhibited the expression of αSMA and pro-collagen 1a1 mRNA in both human (chapter 4) and rat (not shown) liver slices. This inhibitory effect of pentoxifylline on αSMA and pro-collagen 1a1 mRNA expression in liver slices was observed at a time-point at which the expression was not yet increased as compared to expression levels in slices prior to incubation. This suggests that the fibrogenic process in liver slices occurs already early during incubation, but is masked by the simultaneously occurring process that leads to decreased expression of αSMA and pro-collagen 1a1 mRNA.

Taken together, it was concluded that the fibrogenic process that occurs spontaneously in rat and human liver slices could provide a simple system to study fibrogenesis in the liver and to test anti-fibrotic drugs in a multicellular environment closely resembling the *in vivo* situation. However, further elucidation of the processes underlying both the initial decreased expression of stellate cell activation and fibrogenesis markers and their subsequent increased expression after prolonged incubation is necessary to further characterize this model.

3.4 FIBROTIC RAT LIVER SLICES

As discussed above, liver slices could provide an *in vitro* system in which stellate cell activation, fibrogenesis, and the effects of anti-fibrotic compounds can be tested in a milieu that resembles the *in vivo* situation more closely than current *in vitro* models. In addition, when using slices from fibrotic livers for this purpose, this enables the study of progressed stages of fibrosis in a multicellular, pathophysiologic milieu, which cannot be achieved *in vitro* using other models. Besides, these slices may be used to study the processes underlying the resolution of fibrosis and the de-activation of stellate cells. Liver slices generated from fibrotic liver tissue have been used for the study of collagen synthesis, the toxicity of gliotoxin, the binding and uptake of a drug-carrier, and the effects of prostaglandins [81-84]. However, to our knowledge, the applicability of these slices to study liver fibrosis and to test the effects of anti-fibrotic drugs was not previously evaluated. Therefore, we evaluated liver slices generated from fibrotic rat liver tissue as a tool to study fibrosis and test anti-fibrotic compounds (chapter 5).

Fibrotic rat liver slices were generated from rat liver three weeks after bile-duct ligation (BDL). These livers showed clear signs of fibrosis compared to control rat liver, with prominent collagen protein content and proliferated bile duct epithelial cells, which are characteristic for BDL-induced fibrosis [85]. These features were retained after the slicing procedure, indicating that the liver slices still were fibrotic. During incubation up to 48 hours, viability of the fibrotic liver slices and the different liver cell types therein was maintained, with retained cell-cell interactions (chapter 5).

Incubation of fibrotic rat liver slices resulted in similar changes in the mRNA expression of αSMA, pro-collagen 1a1, fibulin-2, and synaptophysin as described above for normal rat liver slices (chapter 5). The significantly decreased expression of αSMA in fibrotic liver slices during the first hours of incubation might be explained by an initial regression of fibrosis. However, as discussed above, a similar initial decrease of αSMA mRNA expression was observed in liver slices from normal rat liver, suggesting that it is not related to the fibrotic process. After prolonged incubation of fibrotic rat liver slices, the expression of αSMA mRNA and protein as well as pro-collagen 1a1 mRNA expression in the liver slices increased, suggesting that further HSC activation and fibrogenesis occurs. Alternatively, the increased expression may reflect activation of portal fibroblasts or an increase in the number of (myo)fibroblasts or HSC in the slice. Indeed, fibulin-2 and synaptophysin mRNA expression showed a similar expression pattern during incubation, indicating that both HSC and (myo)fibroblasts are involved in this process. *In vivo*, both activated hepatic stellate cells as well as other (myo)fibroblast populations are involved in fibrogenesis [58]. In the fibrotic liver slices, the activation and/or proliferation of fibrogenic cells was also accompanied by an

increased production of extracellular matrix proteins as was shown by the increasing collagen protein content during incubation of the liver slices. Since a similar fibrotic process was observed in liver slices from normal rat liver, we concluded that the occurrence of this fibrotic process during incubation in fibrotic liver slices is intrinsically related to the preparation and the culturing of the liver slices rather than being caused by sustained *in vivo* fibrosis.

To evaluate fibrotic rat liver slices as a tool to test anti-fibrotic drugs, the effects of pentoxifylline, gleevec, and dexamethasone on αSMA and pro-collagen 1a1 mRNA expression in the liver slices was studied. All three compounds inhibited the increase in mRNA expression of these markers that occurred after prolonged incubation of the fibrotic slices (chapter 5). This is in line with several previous *in vitro* studies showing that both gleevec and pentoxifylline inhibit proliferation and pro-collagen mRNA expression in cultured hepatic stellate cells and (myo)fibroblasts [72, 73, 80, 86]. As mentioned, this effect of pentoxifylline is generally attributed to inhibitory effects on the PDGF signaling pathway by elevation of intracellular cAMP levels [68]. Gleevec is a protein tyrosine kinase inhibitor with anti-fibrotic effects, which are mainly mediated via the inhibition of the PDGF signaling pathway by preventing auto-phosphorylation of the PDGF receptor [87]. In addition, gleevec may inhibit downstream of the transforming growth factor β signaling (TGFβ) pathway via inhibition of c-Abl tyrosine kinase [88, 89]. Dexamethasone was shown to inhibit pro-collagen 1a1 mRNA synthesis in several studies using stellate cell culture models [90-92], which is in line with its inhibitory effects on pro-collagen 1a1 mRNA expression in fibrotic rat liver slices. However, in cell culture models this inhibition did not result in reduced extracellular matrix synthesis [92], and also αSMA mRNA expression was not inhibited by dexamethasone [91]. In contrast, in fibrotic liver slices, αSMA mRNA expression was significantly inhibited by dexamethasone. This might be explained by the multicellular nature of the fibrotic liver slice, and could be caused by the anti-inflammatory effects of dexamethasone rather than by direct effects on activated stellate cells or other (myo)fibroblasts. This possible indirect inhibitory effect of dexamethasone on hepatic stellate cell activation cannot be measured in single-cell cultures of hepatic stellate cells. *In vivo*, anti-fibrotic effects of dexamethasone were observed in a model for biliary sclerosis [93] and in a schistosomiasis model [94], which was also attributed to its anti-inflammatory effects. In contrast, dexamethasone did not reduce established dimethylnitrosamine-induced fibrosis [95]. Similarly, reports on the anti-fibrotic effects of pentoxifylline in experimental animal models for liver fibrosis are rather contradictory. In the yellow phosphorus model the collagen content of the livers was reduced by pentoxifylline [79, 96]. However, in BDL or carbon tetrachloride-induced liver fibrosis pentoxifylline had only a moderate [97] or no [96, 98, 99] beneficial effect on collagen protein content or fibrotic areas in the livers. In contrast, several studies did report pentoxifylline-induced reduction of hepatic stellate cell activation and proliferation, which is in line with our study, and suppression of TGFβ mRNA expression in these models [74, 80]. Gleevec has anti-fibrotic effects in pig-serum induced fibrosis and in the early phase of BDL-induced fibrosis, but was ineffective in established fibrosis in the BDL-model [86, 100].

Taken together, these results indicated that fibrotic rat liver slices represent a useful tool to test anti-fibrotic compounds. However, a comparison of the results obtained with liver slices and other models for liver fibrosis with respect to the anti-fibrotic effects of the different compounds studied shows that, despite the multicellular, fibrotic milieu in these liver slices, extrapolation to the *in vivo* situation may remain difficult. This may be related to the *in vivo* pharmacokinetics of the compounds studied, or to extrahepatic (side)effects of the compounds, which is not accounted for in *in vitro* models for liver fibrosis. Nevertheless fibrotic liver slices could provide an *in vitro* system in which the effects of anti-fibrotic drugs on stellate cell activation and fibrogenesis can be studied in a multicellular and, importantly, fibrotic environment, which is not possible using other *in vitro* models or normal liver slices.

4 Conclusions

Precision-cut liver slices provide a multicellular *in vitro* model for the liver in which cell-cell and cell-extracellular matrix interactions are maintained. These interactions play an important role in the development of liver fibrosis *in vivo*, but are not adequately incorporated in current available *in vitro* models for liver fibrosis. Therefore, we aimed to evaluate the use of liver slices for the study of fibrosis and as a test system for anti-fibrotic drugs. Our results clearly indicate that liver slices provide a promising alternative for the study of liver fibrosis *in vitro*. Firstly, we have developed a technique to induce early hepatic stellate cell activation via a multicellular mechanism using rat and human liver slices. This model provides a multicellular, physiologic milieu to study the mechanisms underlying toxicity-induced early hepatic stellate cell activation. Secondly, we showed that preparation and/or culturing of rat and human liver slices induced a fibrotic process after prolonged incubation that, similar to the *in vivo* situation, likely involved both activated stellate cells and other myofibroblast populations. This model could provide a simple system to study fibrogenesis in the liver and to test anti-fibrotic drugs in a multicellular environment, closely resembling the *in vivo* situation. Finally, we showed that a similar process occurs during incubation of fibrotic rat liver slices, which provides the unique opportunity to study progressed stages of fibrosis and to test the effects of anti-fibrotic compounds in a fibrotic milieu *in vitro*. The development of this combination of models may contribute substantially to the reduction, refinement, and possible replacement of animal experiments. In addition, the use of human liver tissue in this *in vitro* testing may improve the predictive value of the test results for man.

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